Valproic acid promotes differentiation of hepatocyte-like cells from whole human umbilical cord-derived mesenchymal stem cells

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Mesenchymal stem cells (MSCs) are mesoderm-derived cells that are considered a good source of somatic cells for treatment of many degenerative diseases. Previous studies have reported the differentiation of mesodermal MSCs into endodermal and ectodermal cell types beyond their embryonic lineages, including hepatocytes and neurons. However, the molecular pathways responsible for the direct or indirect cell type conversion and the functional ability of the differentiated cells remain unclear and need further research. In the present study, we demonstrated that valproic acid (VPA), which is a histone deacetylase inhibitor, induced an increase in the expression of endodermal genes including CXCR4, SOX17, FOXA1, FOXA2, GSC, c-MET, EOMES, and HNF-1β in human umbilical cord derived MSCs (hUCMSCs). In addition, we found that VPA is able to increase these endodermal genes in hUCMSCs by activating signal transduction of AKT and ERK. VPA pretreatment increased hepatic differentiation at the expense of adipogenic differentiation. The effects of VPA on modulating hUCMSCs fate were diminished by blocking AKT and ERK activation using specific signaling inhibitors. Together, our results suggest that VPA contributes to the lineage conversion of hUCMSCs to hepatic cell fate by upregulating the expression of endodermal genes through AKT and ERK activation.

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1. Introduction

Human embryonic stem cells (hESCs) and mesenchymal stem cells (MSCs) have ability to self-renew and differentiate into many different cell types. hESCs are derived from the inner cell mass of preimplantation embryos and are capable of giving rise to almost all cell types (Biswas and Hutchins, 2007). We have previously demonstrated that neurons, pancreatic and hepatic cells derived from ESCs improved organ functions after transplantation into animal model of diseases (Kim et al., 2002; Shim et al., 2007; from ESCs improved organ functions after transplantation into animal model of diseases (Kim et al., 2002; Shim et al., 2007; Woo et al., 2012). In contrast hESCs, MSCs, which are derived from fetal or adult mesodermal tissues, have limited proliferative and differentiation capacities and are considered a source of somatic cells for the treatment of several diseases or injuries, including fracture nonunions, osteogenesis imperfecta, hypophosphatasia, and osteoarthritis (Gupta et al., 2012; Undale et al., 2009; Zhang et al., 2012). Recently, human umbilical cord-derived MSCs (hUCMSCs) have received much attention as an ideal cell source for clinical use since they have many advantages, including easy procurement, lower risk of viral contamination and tumor formation, non-invasive accessibility, low immunogenicity, and abundant availability (Heijnen et al., 2012). The umbilical cord harbors different types of MSC-like populations in several compartments, including Wharton’s jelly, the subendothelium of umbilical veins, and the perivascular region, and most previous studies have focused on hUCMSCs derived from entire umbilical cord tissues or from Wharton’s jelly (Can and Karahuseyinoglu, 2007).

Recent studies have expanded application to include the ability to induce differentiation of MSCs in vitro into cells of ectodermal and endodermal origins (Charbord, 2010; Dhinasekaran et al., 2013). In addition, previous studies have reported differentiation of mesodermal MSCs into several endodermal cell types beyond their embryonic lineages, including hepatocytes (Campard et al., 2008; Seo et al., 2005; Talens-Visconti et al., 2006). In general, the differentiation of MSCs into hepatocytes has been induced by serum-free differentiation medium containing several growth factors and cytokines, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), oncostatin M (OSM), and dexamethasone (DEX) (Campard et al., 2008; Seo et al., 2005; Talens-Visconti et al., 2006). However, contrary to embryonic stem cells (ESCs), the hepatic differentiating ability of MSCs is limited with low efficiency. Hepatocyte-like cells derived from MSCs showed low expression levels of mature hepatocyte related genes and proteins. Also, secreted albumin, gamma-glutamyl transpeptidase, and CYP
activity were not detected in previous studies (Campard et al., 2008; Seo et al., 2005).

To overcome these obstacles, many studies have reported the hepatic differentiation using small molecules (less than 5000 molecular weight), including trichostatin A, sodium butyrate, and valproic acid (VPA) as non-viral agents based on their safe and convenient differentiation across a broad lineage spectrum (Chen et al., 2009; Snykers et al., 2007, 2009). VPA is a histone deacetylase inhibitor and is used as an antiepileptic and anticonvulsant drug for various disorders such as schizoaffection diseases, social phobia, and neuropathic pain (Talens-Visconti et al., 2006). In recent reports, VPA significantly improved the in vitro hepatic differentiation of mouse ESCs and mouse bone marrow stromal stem cells (Chen et al., 2009; Dong et al., 2009). These studies demonstrated that VPA treatment could regulate the gene expression patterns of fibroblast growth factor receptors (FGFR-IIIc and FGFR-IIIdc) and c-MET in mouse bone marrow stem cells (BMSCs). The results suggested that the upregulated gene expression of FGFR and c-MET induced by VPA may significantly contribute to hepatic differentiation (Chen et al., 2009). However, the molecular mechanism of VPA on hepatic differentiation from stem cells is not well known and few studies have tested the ability of VPA to differentiate human MSCs into hepatocytes.

The purpose of this study was to investigate the effects of VPA on hepatic differentiation of hUCMSCs and to suggest possible mechanisms of VPA contributing to the lineage conversion from hUCMSCs to hepatic cell fate.

2. Materials and methods

2.1. Isolation and culture of hUCMSCs

Human umbilical cord derived mesenchymal stem cells were obtained from HurimBioCell Inc. after normal deliveries. Tissue collection for research was approved by the institutional review board of HurimBioCell Inc. The cords were drained of all blood and washed in saline with 1% penicillin-streptomycin (GIBCO®, Grand Island, NY, USA). Whole umbilical cord tissues were minced into very fine fragments without removing the vessels. Minced tissues were directly plated in a culture flask. After 7 days, the tissues were removed and the medium was replaced. When cells were confluent, they were subcultured using a trypsin solution. The cells were then seeded into culture flasks in MSC-qualified medium (Invitrogen, MesenPro RSTM medium, Carlsbad, CA, USA) at 1 × 10⁶ cells/cm².

2.2. Treatment of small molecules

Small molecules used in the present study were VPA (Sigma–Aldrich, St. Louis, MO, USA), PD0325901 (PD, Stemgent, Cambridge, MA, USA), LY294002 (LY, Stemgent), MK-2206 (Sellckem, Houston, TX, USA), API-2 (Sigma–Aldrich), bromocriptine (Sigma–Aldrich) and fisetin (Sigma–Aldrich). All small molecules were added in serum-free medium, high-glucose DMEM/F12 supplemented with 1 mM nonessential amino acids (GIBCO®) and 0.1 mM β-mercaptoethanol (Sigma–Aldrich). All hUCMSCs were pretreated with 10 mM VPA, 1 μM PD, 50 μM LY, 2 μM MK-2206, 10 μM API-2, 10 μM bromocriptine and/or 20 μM fisetin for 6 h to induce differentiation.

2.3. Differentiation procedures

2.3.1. Adipogenic and osteogenic differentiation

hUCMSCs at passage 3–5 were plated at a density of 1 × 10⁴ or 3 × 10⁵ cells/cm² in expansion medium for 24 h, and then cultured in STEMPRO® adipocyte and osteocyte differentiation medium (GIBCO®) for 4 weeks. After 4 weeks, lipid vesicles and calcium deposition were evaluated by Oil Red O and Alizarin Red staining. Quantitative analysis of Oil Red O positive cells was measured using a microplate spectrophotometer (520 nm).

2.3.2. Chondrogenic differentiation

hUCMSCs at passage 3–5 were cultured by making droplets of 8 × 10⁶ cells/droplet in culture medium for 24 h. The next day, cell pellets were transferred to suspension dishes and cultured in STEMPRO® chondrogenesis differentiation medium (GIBCO®). Proteoglycans of chondrocytes were stained by Alcian Blue dye after 4 weeks.

2.3.3. Hepatic differentiation

hUCMSCs at passage 3–10 were seeded at a density of 3 × 10⁶ cells/cm² in plates coated with rat tail collagen type I (Sigma–Aldrich) in MesenPro RSTM medium (GIBCO®). Hepatic differentiation was induced by culturing hUCMSCs in ITS medium (Kim et al., 2003). Supplemented with 20 ng/ml hepatocyte growth factor (R&D systems, Minneapolis, MN, USA), 10 ng/ml oncostatin M (Sigma–Aldrich) and 10−6 mol/L dexamethasone (Sigma–Aldrich) for 15 days. Hepatic differentiation was confirmed by immunofluorescence staining with anti-human albumin and Periodic Acid Schiff stain (PAS).

2.3.4. Flow cytometry

Undifferentiated and VPA-treated hUCMSCs were analyzed by flow cytometry for cell surface and cytosolic antigen expression. Undifferentiated cells, at the third passage, were incubated with fluorescent isothiocyanate (FITC)-conjugated antibodies for against monocyte marker (CD14), BD Bioscience, San Jose, CA, USA), B-lymphocyte maker (CD19; BD Bioscience), endothelial cell maker (CD31; BD Bioscience), hematopoietic stem cell marker (CD34; BD Bioscience), lymphocyte marker (CD45; BD Bioscience), mesenchymal stem cell markers, CD44 (BD Bioscience), CD73 (eBioscience, San Diego, CA, USA), CD90 (BD Bioscience), CD105 (eBioscience), and CD146 (BD Bioscience), or mouse immunoglobulin G isotype control (eBioscience) for 30 min at 4°C. VPA treated cells were dissociated and resuspended with PBS with 1% FBS. After washing, the cells were analyzed by FACS-Calibur (BD Bioscience) with FlowJo software (http://www.flowjo.com/; Tree Star Inc., Ashland, OR, USA).

2.4. Determination of cell viability and proliferation

2.4.1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay

For the viability assay, 5 × 10³ cells/well were plated in 96-well plates and cultured with MesenPro RSTM medium for 48 h. The culture medium was changed to serum-free medium with/without VPA (Sigma–Aldrich) for different times (3, 6, 12, 24, 48, 72 h). The medium was then removed and MITT reagent (Sigma–Aldrich) was added to the cells and incubated at 37°C in 5% CO₂ and 95% air. After 2 h incubation, the MITT solution was removed, and 100 μl/well of dimethyl sulfoxide (DMSO; Sigma–Aldrich) was added. The absorbance of the reduced form of MITT was measured at 550 nm and 650 nm in a Microplate Spectrophotometer (Bio-Tek Inc., Winooski, VT, USA).

2.4.2. 5-Ethynyl-2′-deoxyuridine (EdU) incorporation assay

For measuring cell proliferation using EdU experiments, hUCMSCs were seeded at 1.5 × 10³ cells/well in 6-well plates and cultured for a day. Then the medium was changed to serum-free medium containing 10 μM EdU, with or without VPA (Sigma–Aldrich) for 6 h. EdU incorporation was performed and analyzed using the Click-iTH EdU Imaging Kit (Invitrogen). After EdU incorporation, the
images of EdU stained cells were observed using a Zeiss Apotome-Axiovert 200M fluorescent microscope (Carl Zeiss, Obercochen, Germany).

2.4.3. Immunostaining and periodic acid Schiff (PAS) staining

The cells were fixed with cold 4% paraformaldehyde in PBS. Immunostaining was performed using standard protocols, with the primary antibodies used for this study shown in supplementary Table 1. Appropriate fluorescently tagged secondary antibodies (Molecular Probes) or biotin-streptavidin-peroxidase (Vectastatin ABC Elite Kit, Burlingame, CA) were used for visualization. An Apotome-Axiovert 200M (Carl Zeiss) was used for optical sectioning. PAS staining of Hepatocyte-like cells derived from MSCs showed glycogen storage ability characteristic of hepatocytes. Hepatic induced cells were fixed with 10% formalin in 95% cold ethyl alcohol, and then exposed to periodic acid solution (Sigma–Aldrich) for 5 min. After rinsing with distilled water several times, the cells were treated with Schiff's reagent (Sigma–Aldrich) for 15 min at room temperature. The dish was rinsed with differentiated medium and observed using an inverted microscope (Axiovert 40 CFL, Carl Zeiss).

2.4.4. Uptake of low-density lipoprotein (LDL)

Undifferentiated hUCMSCs and differentiated hepatocyte-like cells with/without pretreated VPA were incubated with 15 μM acetylated LDL labeled with 1, 1′-dioctadecyl-10-3,3,30,30-tetramethyl-indo-carbocyanine perchlorate (Dil-Ac-LDL; Invitrogen) for 6 h and then Hoechst 33342 was added for

![Graph](image_url)
nucleic acid staining. LDL-positive cells were visualized using a confocal laser microscope (Carl Zeiss).

### 2.4.5. Biochemical analysis of hepatic function

To evaluate urea and albumin secretion, undifferentiated hUCMSCs and hUCMSCs-derived hepatocyte-like cells were cultured in DMEM/F12 medium supplemented with 0.05% FBS (for the prevention of protein aggregation) for 48 h. Urea content in culture medium was assayed using a commercially available kit (Bioassay systems, Hayward, CA) and secreted albumin was measured by the Human Albumin ELISA Quantification Kit (Bethyl laboratories) according to the manufacturer’s protocol.

### 2.4.6. Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR) analysis

Total RNA was extracted from cells using Trizol (Invitrogen) and cDNA was synthesized from 1 μg of total RNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas Inc., Glen Bunie, ML, USA) according to the manufacturer’s instructions. Subsequent PCR amplification was performed in a 20 μl reaction volume using AccuPower® PCR-Premix (Bioneer, DaeJeon, Republic of Korea). qPCR was also performed using the CFX-96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with IQ SYBR Green Supermix (Bio-Rad). Relative gene expression was normalized to the amount of GAPDH in each sample. Primer sequences used in PCR and qPCR are listed in supplementary Table 2.

### 2.4.7. Transmission electron microscopy

Undifferentiated hUCMSCs and differentiated hepatocyte-like cells with/without pretreated VPA were fixed with 1% OsO₄ in phosphate buffer for 90 min under dark condition. After dehydration in a graded ethanol series, cells were embedded in Epon-812 mixture and cut into 60 nm ultra thin sections (Leica, Germany). The sections were stained with lead citrate and examined with H-7600 transmission electron microscope (Hitachi, Japan).

### 2.4.8. Western blot analysis

Protein samples of cells were prepared in RIPA lysis buffer (Upstate, Lake Placid, NY, USA). A total of 40 μg of protein from each sample was separated by SDS-PAGE (Invitrogen) and transferred to polyvinylidene fluoride membrane transfer membranes (Schleicher & Schull, Dassel, Germany). The membranes were incubated for 60 min with 5% skim milk to block nonspecific antibody binding sites. After blocking, the membranes were immunoblotted with antibodies overnight at 4 °C. Antibodies used in the present study are listed in supplementary Table 1. After washings, the membranes were incubated for 1 h with HRP-conjugated secondary antibody, and washed. Finally, the membranes were developed with the enhanced chemiluminescence (ECL) kit (Westsaveup, abFRONTIER, Anyang, Republic of Korea) to detect bands.

### 2.5. Statistical analysis

Numerical values were expressed as the mean ± SE of three independent experiments performed in triplicate. Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by the Bonferroni’s test procedure for multiple comparisons with the appropriate control. P values less than 0.05 were considered statistically significant.

### 3. Results

#### 3.1. Characterization of hUCMSCs

Undifferentiated hUCMSCs are positive for the surface antigens CD44, CD73, CD90, CD105, and CD146, which are representative of human MSC markers, but were negative for CD14, CD19, CD31, CD34, and CD45 (Fig. 1A). In addition, immunocytochemistry and RT-PCR results showed that hUCMSCs expressed collagen I, HLA-A, CD90, CD73, CD105, CD117, Alkaline phosphatase (ALP), Oct4, and stem cell factor (SCF), but they did not express both CD31 and HLA-A.

![Fig. 2.](image-url) Proliferation and morphological change of hUCMSCs after VPA treatment. (A) Morphology of hUCMSCs observed under a phase contrast microscope after 0 (CON), 2.5, 5, and 10 mM VPA treatment for 6 h. Scale bars: 100 μm. (B) Effects of VPA on the cell viability in hUCMSCs. Cell viability was assessed by MTT assay at 3, 6, 12, 24, 48, 72, and 96 h after exposure to 10 mM VPA. Values were represented as percentage of cell viability compared to the control and expresses as means ± SE of triplicate determinations. (C) Effects of VPA on the proliferation of hUCMSCs. Cell proliferation was assessed by E-dU (green) incorporation assay. Cells were counterstained with DAPI (blue). Scale bars: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
DR (Fig. 1B and C). According to the results of staining with Alizarin Red S, Oil Red O, and Alician Blue, hUCMSCs have multiple differentiation potentials for osteogenic, adipogenic, and chondrogenic lineages (Fig. 1D). The population doubling time of these cells was approximately 2.6 days until 50 days (up to 10 passages), which was comparable with human adipose-derived MSCs (Fig. 1E) (Gruber et al., 2012). These data indicate that hUCMSCs used in the present study retained multiple characteristics of MSCs.

3.2. Proliferation and morphologic change of hUCMSCs after VPA treatment

To examine the cytotoxicity and proliferation of VPA, hUCMSCs grown in serum-free medium were treated with various concentrations and different time periods of VPA. After treatment with 10 mM VPA, hUCMSCs became more flat compared to the control group which showed spindle-shaped cells at 6 hr (Fig. 2A). However, the overall viability of hUCMSCs did not significantly decrease with 10 mM VPA treatment even after 96 h, although there was a momentary decrease in viability after 3 h of treatment (Fig. 2B). In addition, we confirmed that VPA had no effect on proliferation of hUCMSCs, as determined by the rate of E-dU positive cells (% of DAPI) (Fig. 2C). These results showed that 10 mM VPA changed the morphology of hUCMSCs without significantly altering viability and proliferation under our experimental conditions.

3.3. Induction of endoderm-related gene expression by VPA

We next examined the change of endodermal and mesodermal gene expressions in hUCMSCs after treatment with VPA. Compared
with control hUCMSCs, treatment of hUCMSCs with VPA (10 mM) for 6 h enhanced the expression of genes involved in endoderm differentiation such as SOX17, FOXA1, FOXA2, GSC, c-MET, EOMES, and HNF-1B. Interestingly, expression of Nanog, a pluripotency sustaining factor in embryonic stem cells, was also increased by VPA treatment. However, transcript levels of mesodermal genes, PDGFRα and VEGFR2 were either not changed or slightly decreased by VPA treatment (Fig. 3A). In particular, CXCR4, which is a cell surface marker identifying endoderm (King et al., 2008), was significantly increased by VPA in a concentration-dependent manner, as determined by qPCR and RT-PCR (Fig. 3B).

VPA increased the expression of endodermal genes in mesodermal hUCMSCs, we next tested whether these cells had the potential to differentiate into hepatic cells, which are differentiated from the endoderm during early embryonic development. The hUCMSCs were first treated or not with VPA for 6 h and then were differentiated for 15 days in ITS medium containing HGF, OSM, and DEX. The production and secretion of urea and albumin are major functions of normal hepatocytes. After differentiation in the presence of HGF, OSM, and DEX, the level of urea secretion was significantly increased in VPA non-treated hUCMSCs (Fig. 3C, blue bar), compared to undifferentiated hUCMSCs (Fig. 3C, gray bar). In addition, the urea secretion rate was further enhanced in differentiated hUCMSCs by pretreatment with VPA (Fig. 3C, red bar). However, secretion of albumin was not significantly different between the groups (Fig. 3D). We further examined and compared other features of hepatocyte in these cells. Fibroblast-like hUCMSCs became polygonal or round-shape after differentiation (Fig. 3E, left panels). The signals for PAS staining which reacts with glycogen were increased, but intense positive signals were observed only when the cells were pretreated with VPA before differentiation (Fig. 3E, middle panels). In contrast to the results obtained from the albumin secretion assay, the number of cells that were positive for albumin immunostaining was profoundly increased by the pretreatment with VPA (Fig. 3E, right panels). The human LDL complex delivers cholesterol to cells by receptor-mediated endocytosis and most LDL is metabolized in liver cells. Undifferentiated hUCMSCs showed no ability to uptake LDL (Fig. 3F, top panels). However, after differentiation, hUCMSCs were able to uptake LDL and this ability was markedly increased by VPA pretreatment (Fig. 3F middle and bottom panels). Transmission electron microscopy revealed that the number of mitochondria and rough endoplasmic reticulum was increased after differentiation and differentiated hUCMSCs which were pretreated with VPA contained glycogen granules (Fig. 3G). These data suggest that VPA promoted the hepatic differentiation by upregulating expression of endoderm-associated genes in mesodermal hUCMSCs.

### 3.4. Effects of VPA on signal transduction of endodermal induction

To investigate signal transduction pathways after VPA treatment, we examined protein levels of AKT1 and ERK1/2 which are involved in differentiation of MSCs into various cell types (Jaiswal et al., 2000; Pelaez et al., 2012) The expression levels of phospho-AKT1 and ERK1/2 proteins were increased in VPA-pretreated hUCMSCs (Fig. 4A and B). We observed a slight decrease in phospho-GSK-3βser9 and increase in β-catenin, but the changes were not statistically significant (Fig. 4C).

We next determined the relationships between endodermal genes and VPA-increased phospho-AKT1 and ERK1/2 proteins by using specific signaling inhibitors and activators. As expected, the VPA treatment increased the expression of endodermal genes (CXCR4, SOX17, FOXA2, and AFP) and this effect was verified at the protein level by immunofluorescence staining using antibodies against SOX17 and FOXA2 (Fig. 5B and C). PD0325901 (PD), which is a small molecule that inhibits ERK by targeting mitogen-activated protein kinase, significantly decreased the level of phospho-ERK1/2 in hUCMSCs (Fig. 5A). However, the PD-induced inhibition of ERK activation failed to lead to significant decrease in the endodermal induction at the gene and protein levels (Fig. 5B and C), suggesting that ERK pathway may not mediate the VPA-induced endodermal induction. Next, LY294002 (LY), an inhibitor of AKT pathway, decreased the level of phospho-AKT1 in hUCMSCs and there was no significant downregulation of endodermal genes and proteins (Fig. 5A–C). Interestingly, however, a combined treatment of PD and LY clearly decreased the level of endodermal genes and proteins (Fig. 5B and C). LY has been shown to block phosphoinositide 3-kinase (PI3K)-dependent AKT phosphorylation. To investigate the role of AKT pathway more precisely, we tested additional AKT-specific inhibitors MK-2206 and API-2. In both cases, the
endodermal gene expressions were downregulated, only when the cells were treated with these inhibitors, together with PD (Fig. 5D and E). In addition, activation of the both ERK and AKT pathways by treatment with signaling activators (fisetin and bromocriptine, respectively) strongly increased the expression of endodermal gene, SOX17 in the absence of VPA (Fig. 5F and G). Therefore, these results suggested that VPA upregulated endodermal genes in mesodermal hUCMSCs through both ERK and AKT signal transduction pathway.

3.5. Effect of ERK and AKT inhibition on hepatic differentiation of hUCMSCs

To confirm the role of ERK and AKT in hepatic differentiation of hUCMSCs, cells were pre-treated with VPA alone or in combination with inhibitors for 6 h prior to hepatic, adipogenic, osteogenic and chondrogenic differentiation. According to PAS and Oil Red O staining, differentiation of hUCMSCs into hepatocyte-like cells was improved by VPA treatment, but this increased hepatic differentiation was diminished when treated with LY and PD (Fig. 6A and B). Contrary to the improved hepatic differentiation, VPA significantly decreased adipogenic differentiation and this effect was recovered when the cells were treated with a combination of VPA and inhibitors (Fig. 6A and C). However, osteogenic or chondrogenic differentiation of hUCMSCs were not affected by VPA or inhibitors under our experimental conditions.

4. Discussion

There has been recent interest in the development of method to differentiate MSCs for therapeutic use in various types of diseases...
Fig. 6. Effects of VPA and small molecule inhibitors on hepatic, adipogenic, osteogenic, and chondrogenic differentiation. (A) Opposite effects on differentiation of hepatocyte and adipocyte by VPA. hUCMSCs were pretreated or not with VPA, LY, and/or PD for 6h and differentiated under hepatogenic, adipogenic, osteogenic and chondrogenic culture conditions described in Section 2. Differentiation potential of hUCMSCs was evaluated by PAS (hepatogenic), Oil Red O (adipogenic), Alizarin Red (osteogenic) and Alcian Blue staining (chondrogenic). Scale bars: 100 μm. (B) Quantification of PAS staining. PAS positive areas were measured using ImageJ and expressed as means ± SE. (C) Quantification of the Oil Red O staining. Oil Red O (lipid accumulated in differentiated hUCMSCs) was solubilized and the optical density was measured at 520 nm, *p < 0.05.

including those of the liver (Banas et al., 2008). However, hepatic differentiation protocols for MSCs have not yet been defined, and the efficiency of differentiation is insufficient for therapeutic applications. Based on previous studies, the major reason of this low efficiency is that MSCs were originally developed from embryonic mesodermal lineages and thus they have to be differentiated into hepatocytes derived from endodermal lineages, prior to hepatic induction. Here, we report that a small molecule, VPA, increases the expression of endoderm-specific genes in hUCMSCs by activating ERK and AKT signal transduction resulting in enhanced hepatic differentiation.

The initial population of MSCs in our study was derived from whole umbilical cord tissues by mechanical dissociation and explant culture after draining blood. Thus the MSC population might contain heterogeneous MSCs derived from different parts of the umbilical cord, including Wharton’s Jelly and the subendothelium of umbilical cord veins. Our data showed that 80–98% of hUCMSCs used in the present study were positive for CD44, CD73, CD90, and CD105 and negative for CD14, CD19, CD31, CD34, CD45, and HLA-DR. In addition, the hUCMSCs showed continuous proliferative potential and high telomerase activity at least until passage 10, and were able to differentiation into osteoblasts, adipocytes, and chondrocytes in vitro. Thus these results demonstrate that the whole umbilical cord-derived MSCs used in the present study display the characteristics of human MSCs as defined by the international society for cellular therapy (Dominici et al., 2006).

Many previous studies have reported that small molecules can be used to induce differentiation of murine and human MSCs into hepatocytes (Aurich et al., 2009; Chen et al., 2009). However, the mechanism of action of small molecule in endodermal or hepatic differentiation is still poorly understood. Among several small molecules, VPA is a deacetylase inhibitor which has membrane permeability. Thus VPA controls cellular histone acetylation, involved in transcription of cellular processes that significantly improved the efficiency of reprogramming in murine and human induced pluripotent stem cells (Huangfu et al., 2008; Liu et al., 2011). In the present study, VPA increased the expression of endoderm-related genes at the mRNA levels of SOX17, FOXA1, FOXA2, GSC, c-MET, EOMES, and HNF-1β and SOX17 and FOXA2 at the protein level. Furthermore, using qPCR, we confirmed a dose-dependent increase of CXCR4, which is used to identify and purify embryonic endodermal cells (Kopper and Benvenisty, 2012; Takenaga et al., 2007). Previously, investigators have reported that VPA-induced CXCR4 expression required inhibition of histone deacetylases, and involved histone hyperacetylation at the promoter region of the CXCR4 gene (Gottlicher et al., 2001; Gul et al., 2009; Tsai et al., 2010). Therefore, in our study VPA probably increased CXCR4 expression in hUCMSCs by chromatin remodeling.

In mouse BMSCs following VPA treatment, mRNA levels of FGFR-1IIc, FGFR-2IIc, and c-MET were increased and the activation of FGFR/FGF-4 and c-MET/HGF signaling pathways contributed to the VPA-mediated hepatic differentiation though epigenetic modification (Chen et al., 2009). In the present study, we found that VPA activated ERK and AKT signal transduction in hUCMSCs. Furthermore, using specific signaling inhibitors and activators, we confirmed that activated both ERK and AKT were involved in the increase in endodermal gene expressions. Because both ERK and AKT are downstream targets of the HGF signaling pathway, HGF is the major cytokine involved in proliferation, migration and differentiation of MSCs (Forte et al., 2006; Rodrigues et al., 2010). Therefore, it is plausible that ERK and AKT-mediated upregulation of endodermal genes may contribute to VPA-induced hepatic differentiation of hUCMSCs. However, further study is needed to investigate the relationships among c-MET/HGF, ERK, and AKT signal transduction pathways in endodermal or hepatic induction of hUCMSCs.
Signal transduction of ERK and AKT play a critical role in MSC differentiation to the adipogenic, osteogenic or chondrogenic lineage. A previous study showed that attenuation of ERK-stimulated osteogenesis promoted the adipogenic differentiation of BMSCs (Jaiswal et al., 2000). In agreement with this finding, adipogenic differentiation of hUCMSCs was decreased in hUCMSCs by ERK and AKT activation after VPA pretreatment. On the other hand, hepatic differentiation was enhanced in VPA-pretreated hUCMSCs in the expense of adipogenic differentiation but not osteogenic and chondrogenic differentiation. Furthermore, the hepatic differentiation was attenuated while adipogenic differentiation was increased by signaling inhibitors, LY and PD. It remains unclear why only adipogenic but not osteogenic and chondrogenic differentiation was affected by VPA treatment.

Overall, our data suggest that VPA actively shifted the differentiation path of hUCMSCs into the hepatic differentiation through ERK and AKT-mediated upregulation of endodermal genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tice.2013.12.006.

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